TITLE OF THE INVENTION

NEURONAL DIFFERENTIATION OF STEM CELLS

RELATED APPLICATIONS/PATENTS & INCORPORATION BY REFERENCE

This application claims priority to U.S. Application Serial No. 60/484,318, filed July 2, 2003.

Each of the applications and patents cited in this text, as well as each document or reference cited in each of the applications and patents (including during the prosecution of each issued patent; "application cited documents"), and each of the PCT and foreign applications or patents corresponding to and/or claiming priority from any of these applications and patents, and each of the documents cited or referenced in each of the application cited documents, are hereby expressly incorporated herein by reference. More generally, documents or references are cited in this text, either in a Reference List before the claims, or in the text itself; and, each of these documents or references ("herein-cited references"), as well as each document or reference cited in each of the herein-cited references (including any manufacturer's specifications, instructions, etc.), is hereby expressly incorporated herein by reference. Reference is specifically made to PCT application Nos. PCT/US01/02064748, filed on August 4, 2000 (published as WO 01/110011 on February 15, 2001), and PCT/US02/04652 filed on February 14, 2002 (published as WO 02/064748 on August 22, 2002), the contents of which are incorporated herein by reference.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This work was supported by NIH Grants RO1-DK061847, RO1-DK58295. The government may have certain rights to the invention.

FIELD OF THE INVENTION

The present invention relates to compositions and methods for culturing stem cells (e.g., multipotent adult progenitor cells, or "MAPCs"), such that neuronal differentiation is achieved. Other aspects of the invention are described in or are obvious from the following disclosure, and are within the ambit of the invention.

BACKGROUND OF THE INVENTION

Stem cells are capable of forming at least one, and sometimes many, specialized cell types. Non-embryonic stem cells include, for example, neural stem cells, hematopoietic stem cells, endothelial progenitor cells, and mesenchymal stem cells. Until recently, it was thought that such cells were progenitor cells that could only differentiate into cell lineages derived from the tissue of origin (i.e., that hematopoietic stem cell could only differentiate into hematopoietic lineages). However, several recent studies indicate that these cells can differentiate into cells of different lineages (Ferrari, G. et al, 1998; Gussoni, E. et al, 1999; Rafii, S. et al, 1994; Asahara, T. et al, 1997; Lin, Y. et al, 2000; Orlic, D. et al, 2001; Jackson, K. et al, 2001; Petersen, B.E. et al, 1999; Theise, N.D. et al, 2000; Lagasse, E. et al, 2000; Petersen, B.E. et al, 1999; Theise, N.D. et al, 2000; Lagasse, E. et al, 2000; Krause, D.S. et al, 2001; Mezey, E. et al, 2000; Brazelton, T.R. et al, 2000; Orlic, D. et al, 2001; Bjornson, C. et al, 1999; Shih, C.C. et al, 2001; Jackson, K. et al, 1999; Kawada, H. and Ogawa, M., 2001). Multipotent adult progenitor cells, or MAPCs, for example, home to liver, lung, gut and bone marrow and spleen when transfused into murine recipients, where they differentiate in a tissue specific manner.

Despite recent advances in selection techniques, simple, efficient and highly effective culture conditions for use in the differentiation of stem cells into several terminally differentiated cell types have yet to be developed. Neuronal cells are an example of a cell type for which improved methods of differentiation methods are needed. A number of studies have found that terminal neuronal differentiation requires yet-to-be characterized factors secreted by region-specific glial cells. For instance, Wagner et al found that co-culture of Nurr1 neurons with type-II astrocytes from primary E16 rat ventral mesencephalon, the age and region where endogenous neurons of the substantia nigra have just been born, yielded a significant numbers of functioning dopaminergic neurons (Wagner, J. et al, 1999). Panchision et al created a type-II astrocyte line that supports terminal differentiation of dopaminergic neurons (Panchision, D.M. et al, 1999). Song et al also demonstrated that neural differentiation *in vitro* occurred when NSCs were co-cultured with brain-derived astrocytes (Song, H. et al, 2002). With the exception of perhaps neuronal stem cells, culture conditions for stem cells have not been shown to consistently produce

neuronal cells that have undergone developmentally correct progression throughout the in vitro differentiation process (i.e., show morphological and biochemical changes that temporally correspond to those seen in vivo differentiation).

Obtaining neuronal cells suitable for transplantation will have great use in the treatment of neurodegenerative diseases, such as amyotrophic lateral sclerosis, Alzheimer's, and Parkinson's disease. Stem cells, such as MAPCs, can potentially serve as an unlimited source of neuronal tissue for such purposes. Accordingly, culture conditions that achieve differentiation of stem cells to functional neuronal cells that mimics in vivo differentiation would be desirable.

SUMMARY OF THE INVENTION

The present invention relates to methods of culturing stem cells, such that neuronal differentiation can be achieved.

In one aspect, the present invention relates to methods for inducing stem cells to differentiate into neuronal cells comprising sequentially providing specific factors to stem cells in culture. The methods produce cells having undergone biochemical and morphological changes that are characteristic of developing neuronal cells in vivo.

Neuronal cells produced by methods of the present invention have a mature phenotype and display neuronal function. Neuronal cells produced by methods of the present invention include, but are not limited to, dopaminergic, serotonergic and GABA-ergic neurons.

In one embodiment, the present invention relates to a method for inducing stem cells to differentiate into neuronal cells, said method comprising the steps of:

a) culturing stem cells with basic fibroblast growth factor;

b) culturing the cells of step a) with fibroblast growth factor 8 and Sonic Hedgehog;

c) culturing the cells of step b) with brain-derived neurotrophic factor; and

d) co-culturing the cells of step c) with astrocytes.

In one exemplified embodiment, astrocytes are derived from fetal brain.

In one embodiment, co-culturing is in a medium having a supplement comprising insulin, transferrin, selenite, putrescine and progesterone.

In an exemplified embodiment, the co-culturing is in a medium containing N2 supplement®.

At each successive step, culturing is for about 5 to 15 days, most preferably for at least 7 days.

Stem cells include, but are not limited to, embryonic stem cells and adult stem cells, such as multipotent adult progenitor cells (MAPCs).

In one embodiment, the stem cells are maintained in the presence of fibronectin.

Other aspects of the invention are described in or are obvious from the following disclosure, and are within the ambit of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows mouse MAPCs that were cultured sequentially for 7 days with 100ng/mL bFGF, 10ng/mL FGF8 and 100ng/mL SHH, and 10 ng/mL BDNF on fibronectin coated chamber slides. After 7, 10 and 21 days, cells were fixed and stained with antibodies against D7: nestin and Nurr1, followed by secondary Cy5 and Cy3 coupled antibodies respectively; D10: (1) NF200 and GFAP, followed by secondary Cy3 and Cy5 coupled antibodies respectively; and (2) NF200 and MBP, followed by secondary Cy3 and Cy5 coupled antibodies respectively; D21: (1) GABA and DDC, followed by secondary Cy5 and Cy3 coupled antibodies respectively; (2) TrH and TH, followed by secondary Cy5 and Cy3 coupled antibodies respectively; and (3) MAP2AB and Tau, followed by secondary Cy3 and Cy5 coupled antibodies respectively.

Figure 2A shows cells labeled with antibodies against GABA and DDC: Photos 1-3 show single fluorescence color analysis: (1) cells stained with antibodies against GABA followed by secondary Cy3 coupled antibody; (2) eGFP labeled cells; (3) cells stained with antibodies against DDC followed by secondary Cy5 coupled antibody; Photos 4-6 represent overlay pictures: (4) GFP/anti-GABA-Cy3; (5) anti-GABA Cy3/Anti-DDC-Cy5; (6) GFP/anti-DDC-Cy5. Shown are GFP positive cells that have acquired morphological and phenotypic features of GABA-ergic and dopaminergic neurons, whereas a fraction of cells with morphological and phenotypic features of GABA-ergic and dopaminergic neuron was GFP negative.

Figure 2B show cells labeled with antibodies against TrH and Dopamine: Photos 1-3 represent single fluorescence color analysis: (1) cells stained with antibodies against TrH followed by secondary Cy3 coupled antibody; (2) eGFP labeled cells; (3) cells stained with antibodies against dopamine followed by secondary Cy5 coupled antibody; Photos 4-6 represent overlay pictures: (4) GFP/anti-TrH-Cy3; (5) anti-TrH-Cy3/Anti-dopamine-Cy5; (6) GFP/anti-dopamine-Cy5. Shown are GFP positive cells that have acquired morphological and phenotypic features of serotonergic and dopaminergic neurons, whereas a fraction of cells with morphological and phenotypic features of serotonergic and dopaminergic neurons was GFP negative.

Figure 3A shows spiking behavior and voltage-gated currents from MAPCs in co-culture with fetal mouse brain astrocytes. Current-clamp recordings are from a MAPC that had been co-cultured with astrocytes for 8 days. Illustrated in the lower three panels are the voltage responses elicited by the current injection protocol shown (a 17 pA current injection step; top panel). The repetitive spiking recorded in this cell was blocked reversibly by tetrodotoxin (TTX). The current injection protocol reports the current injected relative to a negative DC current which was injected into the cell for the purpose of "holding" it near –100 to –130 mV.

Figure 3B shows voltage-clamp recordings of leak subtracted currents from the same cell as shown in A. The top panel illustrated the voltage-clamp protocol used to elicit the families of currents shown in the lower three panels. A large transient inward current was evident, and this could be blocked reversibly by TTX.

Figure 3C shows current-clamp records obtained from a MAPC that had been co-cultured with astrocytes for 8 days. In this representative, the cell produced only one spike in response to depolarizing current injections (Δ pA = 7). The arrows point to possible synaptic potentials.

Figure 4 depicts the amino acid sequences of basic fibroblast growth factor, fibroblast growth factor 8, Sonic Hedgehog, and brain-derived neurotrophic factor.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the terms below are defined by the following meanings:

"Stem cell" refers to a cell that can give rise to at least two cell types of the ectodermal lineage. A "MAPC" is one type of stem cell. Another is an "embryonic stem cell."

"MAPC" is an acronym for a multipotent adult progenitor cell. It refers to a non-embryonic stem cell that can give rise to cell lineages of all three germ layers upon differentiation. See PCT/US00/21387, published as WO 01/11011, and filed as U.S. Application Serial No. 10/048,757 (specifically incorporated by reference for the description of MAPC isolation, characterization and preparation) and PCT/US02/04652, published as WO 02/064748, and filed as U.S. Application Serial No. 10/467,963 (specifically incorporated by reference for the description of MAPC isolation, characterization and preparation).

"Germ layers" are the three primary layers formed as a result of gastrulation in early stage embryos, consisting of endoderm, mesoderm and ectoderm. Embryonic germ layers are the source from which all tissues and organs derive. The endoderm is the source of, for example, pharynx, esophagus, stomach, intestine and associated glands (e.g., salivary glands), liver, epithelial linings of respiratory passages and gastrointestinal tract, pancreas and lungs. The mesoderm is the source of, for example, smooth and striated muscle, connective tissue, vessels, the cardiovascular system, blood cells, bone marrow, skeleton, reproductive organs and excretory organs. Ectoderm is the source of, for example, epidermis (epidermal layer of the skin), sensory organs, the entire nervous system, including brain, spinal cord, and all the outlying components of the nervous system.

"Multipotent" refers to the ability to give rise to more than one differentiated cell type. MAPCs have extensive multipotency, in that they can give rise to cell lineages of all three germ layers (i.e., endoderm, mesoderm and ectoderm) upon differentiation.

"Progenitor cells" are cells produced during differentiation of a stem cell and have some, but not all, of the characteristics of their terminally differentiated progeny. Defined progenitor cells are committed to a lineage, but not to a specific or terminally differentiated cell type. The term "progenitor" as used in the acronym "MAPC" does not limit these cells to a particular lineage.

"Neuronal differentiation factors" are chemical or biological factors that induce differentiation of stem cells into cells of the neuronal lineage. Neuronal differentiation factors of the invention include, but are not limited to, basic fibroblast growth factor, fibroblast growth factor-8, brain-derived neurotrophic factor, Sonic Hedgehog, N2 supplement®, and combinations thereof that are capable of modulating neuronal differentiation of stem cells in culture.

The terms "comprises", "comprising", and the like can have the meaning ascribed to them in U.S. Patent Law and can mean "includes", "including" and the like.

Methods and Compositions of the Invention

Methods of the present invention induce stem cells in culture to progresses through the appropriate stages of neuronal development, thus recapitulating neuronal development in vitro, and as a result, give rise to cells having functional neuronal properties (e.g., biochemical, anatomical, and electrophysiological characteristics of midbrain neuronal cells).

Culture methods of the invention comprise an ordered addition of neuronal differentiation factors, wherein there is a first addition of basic fibroblast growth factor (Abraham, J. A., 1986); a second addition of fibroblast growth factor 8 (Gemel, J., 1996; Yoshiura, K., 1997) and Sonic Hedgehog (Marigo, V., 1995); a third addition of brain-derived neurotrophic factor (Maisonpierre, P.C., 1991) followed by co-culture with fetal brain astrocytes. Co-culturing can be performed in a medium having a supplement comprising insulin, transferrin, selenite, putrescine and progesterone. In an exemplified embodiment, the co-culturing is in a medium containing N2 supplement®, available from Gibco (Catalog No. 17502048, containing recombinant human insulin, human transferrin (iron-saturated), sodium selenite, putrescine and progesterone in Phosphate Buffered Saline).

Additional components can be added as necessary at each step. For example, PDGF and/or EGF may be present together with bFGF. Similarly, inhibitors of WNT and TGF-β/BMPs (e.g., Noggin) may be present together with bFGF.

At each successive step, the culture is continued for about 5 to 15 days, most preferably for at least 7 days. Optionally, each sequential step comprises only the specified growth factor(s). Medium can be prepared to contain only the growth

factor(s) of interest, and cells can be washed between steps to reduce the presence of previously added growth factor(s). Alternatively, reduced concentrations of the previously provided factor remain in the culture medium.

The amounts of each neuronal differentiation factor can vary, for example, depending on the stem cell selected and the size and duration of the culture. Concentrations can range, for example, between 10-20 ng/mL, 20-30 ng/mL, 30-40 ng/mL, 40-50 ng/mL, 50-60 ng/mL, 60-70 ng/mL, 70-80 ng/mL, 80-90 ng/mL and 90-100 ng/mL. In a specific embodiment, 100 ng/mL bFGF, 10 ng/mL FGF-8, 100 ng/mL SHH and 10 ng/mL BDNF are used. Suitable concentrations can be determined by assaying the differentiation potential of stem cells having undergone the sequential culture methods of the invention.

The neuronal differentiation factors of the present invention are well known in the art. Human basic fibroblast growth factor is described by at least Abraham et al., 1986, the contents of which are incorporated herein by reference. Sequence information for human basic fibroblast growth factor is available as Genbank Accession No. NP_001997 (Figure 4).

Human fibroblast growth factor 8 is described by at least Gemel et al, 1996 and Yoshiura et al, 1997, the contents of which are incorporated herein by reference. Sequence information for human fibroblast growth factor 8 is available as Genbank Accession Nos. P55075, NP_149355, NP_006110, NP_149353, and NP_149354. (Figure 4).

Human Sonic Hedgehog is described by at least Marigo et al, 1995, the contents of which are incorporated herein by reference. Sequence information for human sonic hedgehog is available as Genbank Accession No. Q15465 (Figure 4).

Human brain-derived neurotrophic factor is described by at least Maisonpierre et al, 1991, the contents of which are incorporated herein by reference. Sequence information for human brain-derived neurotrophic factor is available as Genbank Accession No. P23560 (Figure 4).

Methods of the invention contemplate the use of any basic fibroblast growth factor, fibroblast growth factor 8, Sonic Hedgehog, or brain-derived neurotrophic factor known in the art and having conserved function, and from all species (e.g., orthologues from human, mouse, rat, monkey, pig and the like).

Suitable forms of basic fibroblast growth factor, fibroblast growth factor 8, Sonic Hedgehog, or brain-derived neurotrophic factor can comprise isolated polypeptides, that are optionally recombinant, including whole proteins, partial proteins (e.g., domains) and peptide fragments. Fragments of a polypeptide preferably are those fragments that retain the distinct functional capability of the particular factor, which in this case generally relates the ability to influence neuronal differentiation (the specific function of each factor is well known in the art). Such polypeptides can also comprise, for example, fusion proteins and chimeric proteins. Short polypeptides can be synthesized chemically using well-established methods of peptide synthesis.

The invention also contemplates the use variants of neuronal differentiation factors described above (i.e., basic fibroblast growth factor, fibroblast growth factor 8, Sonic Hedgehog, or brain-derived neurotrophic factor). As used herein, a "variant" of a polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence. Variants would include allelic variants and polymorphic variants having conserved function. Modifications which create a polypeptide variant can also be made to 1) enhance a property of a polypeptide, such as protein stability in an expression system or the stability of protein-protein binding; or 2) to provide a novel activity or property to a polypeptide, such as addition of a detectable moiety. Modifications to a polypeptide can be introduced by way of the nucleic acid which encodes the polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the amino acid sequence.

The skilled artisan will also realize that conservative amino acid substitutions may be made in the neuronal differentiation factors described above to provide functionally equivalent variants of the foregoing polypeptides, i.e., the variants retaining functional capabilities. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is

made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York.

Conservative amino-acid substitutions in amino acid sequences typically are made by alteration of the coding nucleic acid encoding. Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a coding gene. Where amino acid substitutions are made to a small peptide fragment, the substitutions can be made by directly synthesizing the peptide. The activity of functionally equivalent fragments of polypeptides can be tested by cloning the gene encoding the altered polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered polypeptide, and testing for a functional capability. Peptides which are chemically synthesized can be tested directly for function.

Stem cell lines and other fastidious cells benefit from co-culturing with another cell type. Such co-culturing methods arise from the observation that certain cells can supply yet-unidentified cellular factors that allow the stem cell to differentiate into a specific lineage or cell type. These cellular factors can also induce expression of cell-surface receptors, some of which can be readily identified by monoclonal antibodies. Generally, cells for co-culturing are selected based on the type of lineage one skilled in the art wishes to induce. Where neuronal differentiation is desired, stem cells of the invention can benefit from co-culturing with glioma, neuroblastoma, oligodendrocyte, microglial, and astrocyte cell-types. Cells available for co-culturing with stem cells are often inactivated by γ-irradiation, similar to feeder cell layers. One embodiment of the present invention uses astrocytes, such as brain-derived astrocytes in co-culture with stem cells. Astrocytes can be obtained from any suitable (or species compatible) source of brain, including fetal or adult brain.

Stem cells can be maintained and allowed to expand in culture medium (i.e., an "initial culture") that is well established in the art and commercially available from the American Type Culture Collection (ATCC). Such media include, but are not limited to, Dulbecco's Modified Eagle's Medium® (DMEM), DMEM F12 medium®, Eagle's Minimum Essential Medium®, F-12K medium®, Iscove's Modified Dulbecco's Medium®, RPMI-1640 medium®. It is within the skill of one in the art to modify or modulate concentrations of media and media supplements as necessary for the stem cells used. It will also be apparent that many media are available as a low-glucose formulation, with or without sodium pyruvate.

Where it is desired for the stem cells to remain in an undifferentiated state, the medium can contain supplements such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), leukemia inhibitory factor (LIF), and combinations thereof. It is apparent to those skilled in the art that supplements that allow the cell to self-renew but not differentiate can be removed from the culture medium prior to differentiation.

In an embodiment specific for MAPCs, supplements are cellular factors or components that allow MAPCs to retain the ability to differentiate into all three lineages. This may be indicated by the expression of specific markers of the undifferentiated state. MAPCs, for example, constitutively express Oct 3/4 (Oct 3A) and maintain high levels of telomerase. Assays for monitoring gene expression are well known in the art (e.g., RT-PCR), and can be conducted using standard methodology.

Also contemplated is supplementation of cell culture medium with mammalian sera. Sera often contain cellular factors and components that are necessary for viability and expansion. Examples of sera include fetal bovine serum (FBS), bovine serum (BS), calf serum (CS), fetal calf serum (FCS), newborn calf serum (NCS), goat serum (GS), horse serum (HS), human serum, chicken serum, porcine serum, sheep serum, rabbit serum, serum replacements, and bovine embryonic fluid. It is understood that sera can be heat-inactivated at 55-65°C if deemed necessary to inactivate components of the complement cascade.

Additional supplements can also be used to supply the stem cells with the necessary trace elements for optimal growth and expansion. Such supplements include insulin, transferrin, sodium selenium and combinations thereof. These

components can be included in a salt solution such as, but not limited to Hanks' Balanced Salt Solution® (HBSS), Earle's Salt Solution®, antioxidant supplements, MCDB-201® supplements, phosphate buffered saline (PBS), ascorbic acid and ascorbic acid-2-phosphate, as well as additional amino acids. Many cell culture media already contain amino acids, however some require supplementation prior to culturing cells. Such amino acids include, but are not limited to, L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-cysteine, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine. It is well within the skill of one in the art to determine the proper concentrations of these supplements.

Antibiotics are also typically used in cell culture to mitigate bacterial, mycoplasmal, and fungal contamination. Typically, antibiotics or anti-mycotic compounds used are mixtures of penicillin/streptomycin, but can also include, but are not limited to amphotericin (Fungizone®), ampicillin, gentamicin, bleomycin, hygromycin, kanamycin, mitomycin, mycophenolic acid, nalidixic acid, neomycin, nystatin, paromomycin, polymyxin, puromycin, rifampicin, spectinomycin, tetracycline, tylosin, and zeocin. Antibiotic and antimycotic additives can be of some concern, depending on the type of work being performed. One possible situation that can arise is an antibiotic-containing media wherein bacteria are still present in the culture, but the action of the antibiotic performs a bacteriostatic rather than bacteriocidal mechanism. Also, antibiotics can interfere with the metabolism of some cell types.

Hormones can also be used in cell culture and include, but are not limited to D-aldosterone, diethylstilbestrol (DES), dexamethasone, β-estradiol, hydrocortisone, insulin, prolactin, progesterone, somatostatin/human growth hormone (HGH), thyrotropin, thyroxine, and L-thyronine.

Lipids and lipid carriers can also be used to supplement cell culture media, depending on the type of cell and the fate of the differentiated cell. Such lipids and carriers can include, but are not limited to cyclodextrin (α, β, γ), cholesterol, linoleic acid conjugated to albumin, linoleic acid and oleic acid conjugated to albumin, unconjugated linoleic acid, linoleic-oleic-arachidonic acid conjugated to albumin,

oleic acid unconjugated and conjugated to albumin, among others. One embodiment uses linoleic acid conjugated to albumin.

Also contemplated is the use of feeder cell layers. Feeder cells are used to support the growth of fastidious cultured cells, including stem cells. Feeder cells are normal cells that have been inactivated by γ-irradiation. In culture, the feeder layer serves as a basal layer for other cells and supplies important cellular factors without further growth or division of their own. Examples of feeder layer cells are typically human diploid lung cells, mouse embryonic fibroblasts, Swiss mouse embryonic fibroblasts, but can be any post-mitotic cell that is capable of supplying cellular components and factors that are advantageous in allowing optimal growth, viability, and expansion of stem cells. In many cases, feeder cell layers are not necessary to keep the stem cells in an undifferentiated, proliferative state, as leukemia inhibitory factor (LIF) has anti-differentiation properties. Often, supplementation of a defined concentration of LIF is all that is necessary to maintain stem cells in an undifferentiated state.

Cells in culture can be maintained either in suspension or attached to a solid support, such as extracellular matrix components. Stem cells often require additional factors that encourage their attachment to a solid support, such as type I and type II collagen, chondroitin sulfate, fibronectin, "superfibronectin", and fibronectin-like polymers, gelatin, poly-D and poly-L-lysine, thrombospondin, and vitronectin. One embodiment utilizes fibronectin.

Neuronal cells produced by methods of the present invention have a mature phenotype and display neuronal function. Monitoring the progress of neuronal differentiation can involve, for example, screening for expression of genetic markers of neuronal differentiation. Developmental progression of the cells in culture can be monitored, for example, by measuring levels of neuroectodermal transcripts including, but not limited to, mRNA for c-Ret, sox1, otx2, otx1, pax2, pax5, and Nurr1, nestin, GFAP, MBP, NF200, Dopamine, TH, GABA, TrH, and DBH. Assays for monitoring gene expression are well known in the art (e.g., RT-PCR), and can be conducted using standard methodology.

Methods of identifying and subsequently separating differentiated cells from their undifferentiated counterparts can be carried out by methods well known in the art. Cells that have been induced to differentiate using methods of the present

invention can be identified by selectively culturing cells under conditions whereby differentiated cells outnumber undifferentiated cells. Similarly, differentiated cells can be identified by morphological changes and characteristics that are not present on their undifferentiated counterparts, such as cell size, the number of cellular processes (i.e. formation of dendrites and/or branches), and the complexity of intracellular organelle distribution. Also contemplated are methods of identifying differentiated cells by their expression of specific cell-surface markers such as cellular receptors and transmembrane proteins. Monoclonal antibodies against these cell-surface markers can be used to identify differentiated cells. Detection of these cells can be achieved through fluorescence activated cell sorting (FACS), and enzyme-linked immunosorbent assay (ELISA). From the standpoint of transcriptional upregulation of specific genes, differentiated cells often display levels of gene expression that are different from undifferentiated cells. Reverse-transcription polymerase chain reaction, or RT-PCR, can also be used to monitor changes in gene expression in response to differentiation. Whole genome analysis using microarray technology can also be used to identify differentiated cells. In the case of neural cells, patch-clamp electrophysiology is another method by which differentiated cells can be advantageously identified.

Accordingly, once differentiated cells are identified, they can be separated from their undifferentiated counterparts, if necessary. The methods of identification detailed above also provide methods of separation, such as FACS, preferential cell culture methods, ELISA, magnetic beads, and combinations thereof. One embodiment of the invention envisions the use of FACS to identify and separate cells based on cell-surface antigen expression.

Compositions and methods of the present invention can result in neuronal cell lines or neuronal cell populations suitable for administration into a mammalian host. The neuronal cell population can be engrafted into specific locations of the central and peripheral nervous system (preferably brain or spinal cord), such that the function of a cell or organ, defective due to injury, genetic disease, acquired disease or iatrogenic treatments, is augmented, reconstituted or even provided for the first time.

Stem Cells of the Present Invention

In general, stem cells of the present invention comprise those having the capacity for neuronal differentiation.

In one embodiment, the stem cells are MAPCs (Jiang, Y. et al, 2002). MAPCs derived from human, mouse, rat or other mammals appear to be the only normal, non-malignant, somatic cell (i.e., non-germ cell) known to date to express very high levels of telomerase even in late passage cells. The telomeres are extended in MAPCs and they are karyotypically normal. Because MAPCs injected into a mammal can migrate to and assimilate within multiple organs, MAPCs are self-renewing stem cells. As such, they have utility in the repopulation of organs, either in a self-renewing state or in a differentiated state compatible with the organ of interest. They have the capacity to replace cell types that could have been damaged, died, or otherwise might have an abnormal function because of genetic or acquired disease.

Human MAPCs are described in U.S. Application Serial No. 10/048,757 (see page 8, lines 23-32; p.9, lines 1-22; p.21, lines 19-32; p.22, lines 1-27; p.25, lines 20-31; pages 26 through p.28, lines 1-13, 20-25; p.29, lines 1-21) and U.S. Application Serial No. 10/467,963 (see p.9, lines 29-32; p.10, lines 1-25), specifically incorporated by reference for the characterization of MAPCs.

Methods of MAPC isolation are described in U.S. Application Serial No. 10/048,757 (p.10, lines 17-32; p.11, lines 1-12; p.22, lines 29-32; p.23, lines 1-32; p.24, lines 1-28; p.71, lines 28-32; p.72 through p.74, lines 1-27) and U.S. Application Serial No. 10/467,963 (p.26, lines 13-34; p.27 through p.28, lines 1-27), specifically incorporated by reference for the methods of isolation described. Methods of MAPC culture are also described in U.S. Application Serial No. 10/048,757 (p.23, lines 25-32) and U.S. Application Serial No. 10/467,963 (p.26, lines 18-29), specifically incorporated by reference for the culture methods described.

Stem cells used in the present invention can also include embryonic stem cells (Lebkowski, J.S. et al, 2001). The quintessential stem cell is the embryonic stem (ES) cell, as it has unlimited self-renewal and pluripotent differentiation potential (Thomson, J. *et al.* 1995; Thomson, J.A. *et al.* 1998; Shamblott, M. *et al.* 1998; Williams, R.L. *et al.* 1988; Orkin, S. 1998; Reubinoff, B.E., *et al.* 2000). These cells are derived from the inner cell mass (ICM) of the pre-implantation

blastocyst (Thomson, J. *et al.* 1995; Thomson, J.A. *et al.* 1998; Martin, G.R. 1981), or can be derived from the primordial germ cells from a post-implantation embryo (embryonal germ cells or EG cells). ES and/or EG cells have been derived from multiple species, including mouse, rat, rabbit, sheep, goat, pig and, more recently, from human and non-human primates (U.S. Patent Nos. 5,843,780 and 6,200,806).

Stem cells of the present invention also include those known in the art that have been identified in organs or tissues (non-embryonic stem cells), such as neural stem cells (NSCs) (Gage F.H. 2000; Svendsen C.N. *et al*, 1999; Okabe S. *et al.* 1996). Several studies in rodents, and more recently, non-human primates and humans, have shown that stem cells persist in adult brain. These stem cells can proliferate *in vivo* and continuously regenerate at least some neuronal cells *in vivo*. When cultured *ex vivo*, NSCs can be induced to proliferate, as well as to differentiate into different types of neurons and glial cells. When transplanted into the brain, NSCs can engraft and generate neural cells and glial cells.

NSCs have been identified in the sub-ventricular zone (SVZ) and the hippocampus of the adult mammalian brain (Ciccolini *et al.*, 1998; Morrison *et al.*, 1999; Palmer *et al.*, 1997; Reynolds and Weiss, 1992; Vescovi *et al.*, 1999) and can also be present in the ependyma and other presumed non-neurogenic areas of the brain (Doetsch *et al.*, 1999; Johansson *et al.*, 1999; Palmer *et al.*, 1999). Fetal or adult brain-derived NSCs can be expanded *ex vivo* and induced to differentiate into astrocytes, oligodendrocytes and functional neurons (Ciccolini *et al.*, 1998; Johansson *et al.*, 1999; Palmer *et al.*, 1999; Reynolds *et al.*, 1996; Ryder *et al.*, 1990; Studer *et al.*, 1996; Vescovi *et al.*, 1993). *In vivo,* undifferentiated NSCs cultured for variable amounts of time eventually differentiate into glial cells, GABAergic and dopaminergic neurons (Flax *et al.*, 1998; Gage *et al.*, 1995; Suhonen *et al.*, 1996). The most commonly used source of NSCs is allogeneic fetal brain. Alternatively, NSCs could be harvested from the autologous brain.

Stem cells of the present invention also include hematopoietic stem cells (HSCs). Bone marrow derived HSCs transplanted into mice incapable of developing cells of myeloid and lymphoid lineages migrated to brain and differentiated into cells that express neuron-specific antigens (Mezey, E. et al, 2000).

Yet another stem cell of the present invention is the mesenchymal stem cell (MSC), initially described by Fridenshtein (1982). A number of MSCs have been

isolated. (See, for example, Caplan, A., *et al.,* U.S. Patent No. 5,486,359; Young, H., *et al.,* U.S. Patent No. 5,827,735; Caplan, A., *et al.,* U.S. Patent No. 5,811,094; Bruder, S., *et al.,* U.S. Patent No. 5,736,396; Caplan, A., *et al.,* U.S. Patent No. 5,837,539; Masinovsky, B., U.S. Patent No. 5,837,670; Pittenger, M., U.S. Patent No. 5,827,740; Jaiswal, N., *et al.,* 1997,; Cassiede P., *et al.,* 1996; Johnstone, B., *et al.,* 1998; Yoo, *et al.,* 1998; Gronthos, S., 1994).

MSCs can also differentiate into ectodermal lineages, including neural, lineages (reviewed in Minguell, J.J. et al, 2001). Woodbury et al determined that bone marrow stromal cells of rodent and human origin can be induced to differentiate exclusively into neurons under certain conditions (Woodbury, D. et al, 2000). Studies by Sanchez-Ramos and coworkers also reported that human and mouse bone marrow stromal cells, when cultured in the presence of EGF or BDNF expressed nestin protein and mRNA, as well as glial fibrillary acidic protein, and the neuron-specific NeuN, all of which are markers for neural precursors (Sanchez-Ramos, J. et al, 2000).

The present invention is additionally described by way of the following illustrative, non-limiting Examples that provide a better understanding of the present invention and of its many advantages.

EXAMPLES

Example 1: Differentiation of multipotent adult progenitor cells (MAPC)

Bone marrow was collected from the femurs of 3-4 week-old 129 x C57BL/6J ROSA26 mice according to guidelines from the University of Minnesota Institutional Animal Care and Use Committees. MAPCs were generated as described previously in Jiang and coworkers (Jiang, Y. et al, 2002). To demonstrate that cells were MAPCs, they were induced to differentiate to endothelium and hepatocyte-like cells as described (Schwartz, R.E. et al, 2002; Jiang, Y. et al, 2002). In addition, these cell populations were shown to contribute to most somatic cells of the mouse following blastocyst injection (Jiang, Y. et al, 2002).

MAPCs induced to differentiate into neuroectodermal lineage were cultured in base medium consisted of 60% Dulbecco's Modified Eagle's Medium-LG (Gibco-BRL, Grand Island, NY), 40% MCDB-201 (Sigma Chemical Co, St Louis, MO) with 1X insulin-transferrin-selenium (ITS), 1X linoleic-acid-bovine-serum-albumin (LA-BSA), 10^{-9}M dexamethasone (Sigma) and 10^{-4}M ascorbic acid 2-

phosphate (Sigma), 100 U penicillin and 1,000 U streptomycin (Gibco, Grand Island, NY), on fibronectin (FN) (Sigma). In some instances, N2 supplement (Gibco) was also added. Cytokines that were added included 100 ng/mL basic fibroblast growth factor (bFGF), 100 ng/mL Sonic Hedgehog, 10 ng/mL FGF8, and 10 ng/mL BDNF (all from R&D Systems, Minneapolis, MN; all product literature relating to the same is herein incorporated by reference)

Astrocytes were prepared according to the following method. Mouse brain was dissected from e16 fetuses in Hanks Balanced Salt Solution (HBSS, Sigma). The dissected brain was minced and incubated in 0.125% trypsin/0.05% DNase (Sigma) in HBSS at 37°C for 20 minutes. The tissue was triturated with a pipette and dissociated to a mixture of single cells and small cellular aggregates. After passing through a 70μm nylon mesh, the astrocytic cells were centrifuged at 1000 rpm for 5 min and resuspended in DMEM +10% fetal bovine serum (FBS; Hyclone, Logan, UT). Astrocytic cells were plated onto culture dishes, pre-coated with poly-D-lysine (100 μg/mL, Sigma) at 4°C overnight, at a density of 600,000 cells/cm^2 until confluent.

After astrocytes had been cultured in DMEM +10% FBS for 8 days, culture medium was switched to serum free medium, supplemented with N2 supplement. Three days later, medium was collected as astrocyte conditioned medium.

MAPC-derived neurons were co-cultured with fetal brain astrocytes. Glass coverslips were coated with 500 μg/ml poly-D-lysine overnight at 4°C. Fetal brain astrocytes that were cultured for 8 days were trypsinized and re-plated on glass coverslips, and then allowed to grow to confluency. Once confluent, the coverslips were evaluated for presence of neurons by staining with antibodies against neurofilament (NF)-200 (see methods below) or placed upside down in cultures of MAPC-derived neuron-like cells. Cultures were maintained in serum free medium, supplemented with N2 supplement and without additional cytokines for 5 – 12 days.

Quantitative-RT-PCR (Q-RT-PCR) was performed to detect changes in levels of neuronal transcription factors and genes. RNA was extracted from MAPC differentiated for 5, 7, 10, 14, and 21 days, and brains from e18 or adult mice using the RNeasy kit (Qiagen, Valencia, CA). Two sequential steps of DNase (Invitrogen, Carlsbad, CA) treatment eliminated contaminating DNA. The resultant mRNA was reverse transcribed and cDNA underwent 40 rounds of amplification (ABI PRISM

7700, Perkin Elmer/Applied Biosystems) under the following reaction conditions: 40 cycles of a two step PCR (95°C for 15 seconds, 60°C for 60 seconds) after initial denaturation (95° C for 10 minutes) with 2 μl of DNA solution, 1X SYBR Green PCR Master Mix reaction buffer (Applied Biosystems). Controls consisted of amplifications without reverse transcription and reactions without addition of cDNA template. Authenticity and size of PCR products were confirmed by melting curve analysis (using software provided by Perkin Elmer) and gel analysis. Primers used and size of expected products are shown in Table 1. mRNA levels were normalized using GAPDH as housekeeping gene, and compared with levels in e18 or adult mouse brain.

Table 1: Primers used for Q-RT-PCR

Gene	Forward	Reverse	Size
Sox-1	AAGATGCACAACTCGGAGATCAG	TGTAATCCGGGTGTTCCTTCAT	51 bp.
Otx-2	CCATGACCTATACTCAGGCTTCAGG	GAAGCTCCATATCCCTGGGTGGAAAG	211 bp.
Otx-1	AGGCGCTGTTCGCAAAGA	CCTCCTCGCGCATGAAGAT	50 bp.
Pax-2	CCAGGCATCAGAGCACATCA	CGTCTGTGTGCCTGACACATT	141 bp.
Pax-5	AAACGCAAGAGGGATGAAGGT	AACAGGTCTCCCCGCATCT	100 bp.
Ptx-3	TGTGTGGCACCTGAGTTCA	CACCCTCAGGAACAGAGTGACTT	107 bp.
CRet	GAGGAAATGTACCGTCTGATGCT	TCTTGACCATCATCTTCTCCAGATC	102 bp.
Nurr-1	TGAAGAGAGCGGAGAAGGAGATC	TCTGGAGTTAAGAAATCGGAGCTG	255 bp.
Nestin	GAGAAGACAGTGAGGCAGATGAGTTA	GCCTCTGTTCTCCAGCTTGCT	113 bp.
GFAP	GAGGAGTGGTATCGGTCTAAGTTTG	GCCGCTCTAGGGACTCGTT	165 bp.
MBP	GTGCAGCTTGTTCGACTCCG	ATGCTCTCTGGCTCCTTGGC	153 bp.
GABA	AGGTTGACCGTGAGAGCTGAAT	TGGGCAGGCATGGGC	68 bp.
DAT	GCAATCATCACCACCTCCATTA	ATGGGCACATTGTGCTTCTG	100 bp.
TH	AGTTCTCCCAGGACATTGGACTT	ACACAGCCCAAACTCCACAGT	100 bp.
TrH	GGATGGAGTCTGATGTCACCAA	TGACGTTTCTCAGGCATTAAGC	120 bp.
DBH	TTCCAATGTGCAGCTGAGTC	GGTGCACTTGCTTGTGCAGT	242 bp.

Cells were characterized for neuronal markers using immunophenotypic analysis. Cells were fixed with 4% paraformaldehyde (Sigma) for 4 minutes at room temperature, followed by methanol (Sigma) for 2 minutes at -20°C. For nuclear ligands, cells were permeabilized with 0.1 Triton-X-100 (Sigma) for 10 minutes. Slides were incubated sequentially for 30 minutes each with primary antibody, and fluorescein (FITC), Cy3 or Cy5 coupled anti-mouse-, goat- or rabbit-IgG antibodies. Between each step, slides were washed with PBS+1%BSA (Sigma). Cells were examined by confocal fluorescence microscopy (Confocal 1024 microscope; Olympus AX70, Olympus Optical Co. LTD, Japan). To assess the frequency of different cell types in a given culture, the number of cells staining positive with a given Ab were counted in four visual fields (50-200 cells per field).

Antibodies against myelin basic protein (MBP)(1:20), NF-200 (1:400), MAP2AB (1:400); tyrosine hydroxylase (TH; 1:1000), dopa-decarboxylase (DDC; 1:100), tryptophan hydroxylase (TrH; 1:250), gamma-aminobutyric acid (GABA; 1:500), control-mouse, -rabbit or -rat IgGs and FITC- or Cy3-labeled secondary Abs were from Sigma. Antibodies against Nestin (1:150) and Nurr1 (1:250) were from BD Transduction Laboratories (Lexington, KY). Antibodies directed against glial fibrillary acidic protein (GFAP, 1:400) were from DAKO Corporation (Carpinteria, CA) or Santa Cruz Biotechnology Inc (Santa Cruz, California). Anti-dopamine antibodies (1: 2000) were from Abcam Limited (Cambridge, UK). Polyclonal antibodies against Tau (1:400) were from Santa Cruz Biotechnology Inc. Cy5-labeled secondary antibodies were from Chemicon International (Temecula, CA).

Undifferentiated mMAPCs did not have neuroectodermal characteristics. No staining was seen with antibodies against nestin, GFAP, NF200, MBP, or neurotransmittors (not shown). By Q-RT-PCR, mMAPC did express low levels of c-Ret, Otx-2, and nestin mRNA, but no mRNA for sox1, otx2, Pax2, Pax5, Nurr1, GFAP, MBP, Dopamine, TH, GABA, TrH, or DBH (Table 2; '1' refers to mRNA isolated from fetal brain, while '2' refers to mRNA isolated from adult brain).

Table 2: mRNA levels on day 5, 7, 10, and 14 of differentiation of mMAPC to neuroectoderm

Gene	Day 0	Day 5	Day 7	Day 10	Day 14
Sox-1[(1)]	0	0.57; 1.79; 2.22	0.58; 1.15; 2.14	0.68; 0.59; 5.58	0.9; ND; 1.74
Otx-2[(1)]	0	0.11; 0.25; 3.4	0.21; 0.06; 4.2	0.12; 0.08; 2.4	ND; ND; 3.5
Otx-1[(1)]	0.01	0.33; 1.18; 7.06	0.36; 0.69; 32.1	0.26; 0.33; 24	1.67; ND; 5.31
Pax-2[(1)]	0	6.0; 8.2; 3.07	4.47; 7.06; 5.43	2.39; 1.64; 2.05	5.5; ND; 4.99
Pax-5[(1)]	0	0.11; 0.14; 0.9	0.13; 0.16; 3.75	0.05; 0.03; 1.84	0.48; ND; 1.33
En-1[(1)]	4.87	0.59; 1.45; 4.36	0.2; 0.8; 11.4	0.15; 0.32; 8	0.4; ND; 1.08
cRet[(2)]	0.14	2.84; 5.46; 15.7	2.19; 5.08; 72.8	2.53; 4.47; 54.2	24,17; ND; 36.9
Nurr-1[(2)]	0	0.55; 2.4; 0.38	1.12; 1.47; 1.11	1.41; 3.59; 1.6	ND; ND; 1.68
Nestin[(1)]	0.52	27.3; 98.0; 50.4	10.2; 88.6; 70.8	5.9; 12.2; 243	6.48; ND; 14.7
GFAP[(2)]	0	0.56; 0.52; 1.05	0.36; 0.52; 3.26	1.32; 5.76; 21.0	11.75; ND; 9.19
MBP[(2)]	0	0.004; 0.006; 0.013	0.003; 0.008; 0.006	1.7; 4.08; 1.8	1.8; ND; 2.33
GABA[(2)]	0	1.36; 1.89; 6.23	0.99; 1.74; 17.5	7.8; 19.77; 94.0	61.8; ND; 69.6
DAT[(2)]	0	1.58; 4.39; 19.9	0.5; 2.83; 22.71	1.81; 5.66; 119	13.4; ND; 23.67
TH[(2)]	0	0.67; 1.31; 0.36	0.5; 1.2; 2.86	1.27; 3.94; 2.54	1.7; ND; 2.29
TrH [(2)]	0	0.49; 1.6; 0.46	0.51; 1.04; 4.99	1.56; 3.56; 7.14	3.92, ND; 3.81
DBH[(1)]	0	0	0	0	0

As neuroprogenitors can be expanded with PDGF-BB and induced to differentiate by removal of PDGF and addition of bFGF (Palmer, T.D. et al, 1999), undifferentiated MAPC were re-plated at 10,000 cells/cm^2 on FN coated plates or chamber slides, removed EGF, PDGF and LIF, but added 100 ng/mL bFGF. Q-RT-PCR of mMAPC treated with bFGF for 5 and 7 days demonstrated acquisition of neuroectodermal transcripts. On day 5 and 7, mRNA for sox1, otx2, otx1, pax2, pax5, and Nurr1 could be detected at levels between 0.1 and 7 fold those seen in fetal brain (n=3) (Table 2). By day 5 and 7, nestin mRNA levels increased to between 7 and 100 fold those in fetal brain (Table 2). Immunohistochemistry showed that by day 5, cells started to express nestin protein. By day 7, 65 ± 11 % of cells stained positive for nestin, 23 ± 8 % percent of nestin positive cells also expressed Nurr1 (n=3)(representative example shown in Figure 1). By day 10, 62±7% of cells expressed NF200, 15±5% GFAP and 11±3 % MBP (n=3)(representative example shown in Figure 1), consistent with the finding that mRNA for GFPA and MBP increased to 1-4 fold over that detected in fetal brain. Double immunohistochemistry showed that GFAP, MBP, and NF200 were never detected in the same cells.

When mMAPC were cultured sequentially with 100 ng/mL bFGF for 7 days, followed by a combination of 10 ng/mL FGF-8 and 100 ng/mL SHH for 7 days and finally 10 ng/mL BDNF for 7 days, the latter in medium also supplemented with N2 medium, a more mature phenotype was seen. Q-RT-PCR demonstrated that by day 10 and 14 levels of GABA, dopamine and TH, and TrH mRNA increased between 1.7 and 120 fold. Immunophenotypic analysis on day 21 showed that 25±7% of cells expressed markers of dopaminergic neurons (shown DDC and TH; also dopamine), 18 ±3% of serotonergic (TrH) and 52±5% of GABA-ergic (GABA) neurons (n=3)(representative example shown in Figure 1). Neuron-like cells became polarized, as Tau and MAP2AB were expressed in axonal and somatodendritic compartments respectively. Fewer than 10% of cells stained positive for astrocytes or oligodendrocyte markers (not shown). Consistent with the immunohistological analyses, the levels of MBP and GFAP mRNA decreased by d21 (not shown).

Example 2: Maturation of murine multipotent adult progenitor cells

Murine MAPCs were seeded at a density of 10^5 cells/well in 6-well tissue culture plates in 1 ml of MAPC culture medium, 1 ml of supernatant of the 293 cell line transfected with a third generation, VSV-g pseudotyped e-GFP-expressing lentivirus (10^7 infectious particles/mL) (a kind gift from Dr. Thierry Vandendriesche, Katholieke Universiteit Leuven, Belgium) and polybrene (8μg/mL final concentration), was added to the wells. After 6 hours of incubation at 37°C and 5% CO_2, the medium was replaced with fresh MAPC medium. Transduction of MAPCs was repeated 3 times. Transduction efficiency of the final population was 28% as determined by counting 200 cells.

Based on studies by Wagner et al (Wagner, J. et al, 1999) and Song et al (Song, H. et al, 2002), we next tested whether cultured neuron-like cells could be maintained *in vitro* for more extended periods of time to allow further maturation, when cultured in the presence of fetal brain astrocytes. Astrocytes were cultured from e16 fetal brain in 10%FCS. After several passages, no neural cells could be detected by immnofluorescence microscopy (not shown). In initial studies, astrocyte conditioned medium was added to the developing neuroectodermal cells generated from MAPC. However, no significant further morphologic maturation was observed (n=2)(not shown). In subsequent experiments, astrocytes were plated onto coverslips and allowed to grow to confluence. Coverslips were placed upside down in chamberslides in which eGFP-transduced MAPC had been cultured for 7 days with bFGF, 7 days with FGF8+SHGH and 7 days with BDNF in N2 medium. After an additional 5-12 days in culture, eGFP-expressing MAPC-derived neuron-like cells were again evaluated by immunofluorescence and we demonstrated that eGFP positive cells, continued to express markers of dopaminergic neurons (~25% TH and dopamine), serotonergic neurons (~25%, TrH) and GABA-ergic neurons (~50% GABA) and acquired a much more mature neural morphology with a more elaborate array of axons (n=3; representative example shown in Figure 2).

eGFP transduced mouse MAPC (28% transduction efficiency) were cultured on fibronectin coated chamber slides sequentially for 7 days with 100 ng/mL bFGF, 10 ng/mL FGF8 and 100 ng/mL SHH, 10 ng/mL BDNF, and finally, with e16 fetal mouse brain astrocytes plated on coverslips that were placed upside down in the chamber slides. After a total of 28 days, cells were fixed and stained. Slides were

analyzed for presence of GFP positive cells, and cells co-staining with Cy3 or Cy5 labeled antibodies.

Example 3: Patch-clamp electrophysiology of differentiated multipotent adult progenitor cells

Standard, whole-cell patch-clamp recording methodologies were used to examine the physiological properties of cultured bone marrow stem cells. Voltage-clamp and current-clamp recordings were obtained using a Dagan 3900A patch-clamp amplifier (Dagan Corporation, Minneapolis), that was retrofitted with a Dagan 3911 expander unit. Patch pipettes, made from borosilicate glass, were pulled on a Narishige pipette puller (model PP-83). The pipettes were filled with an intracellular saline which consisted of the following (in mM): 142.0 KF, 7.0 Na_2SO_4, 3.0 $MgSO_4$, 1.0 $CaCl_2$, 5.0 HEPES, 11.0 EGTA, 1.0 glutathione, 2.0 glucose, 1.0 ATP (magnesium salt), 0.5 GTP (sodium salt)(Sigma). For most recordings, the fluorescent dye 5,6-carboxyfluorescein (0.5 mM; Eastman Kodak Chemicals) was also added to the pipette solution for the purpose of confirming visually, using fluorescence microscopy, that the whole-cell patch recording configuration had been achieved. Pipette resistances ranged from 11 to 24 MOhm. The standard extracellular recording saline was comprised of the following (in mM): 155 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose (Sigma).

For some experiments 1μM tetrodotoxin (TTX) was added to the standard control solution. The pH of the intracellular and extracellular recording solutions was adjusted to 7.4 and 7.8, respectively. Unless otherwise noted, all chemical compounds were obtained through the Sigma (St. Louis) chemical company. PClamp 8.0 (Axon Instruments, Foster City) was used to run experiments, and to collect and store data. The data presented herein were corrected for a 8.4 mV liquid junctional potential, which was calculated using the JPCALC software package (Barry, P.H., 1994). Off-line analyses and graphical representations of the data were constructed using Clampfit 8.0 (Axon Instruments, Foster City) and Prism (Graphpad, San Diego).

Patch-clamp recordings were obtained from 50 MAPCs from 5 independent cultures. Recordings were made from cells that were cultured for 7 days each with bFGF, FGF8b and SHH, and BDNF followed by either co-culture with astrocytes

for 5 (n=9), 7 (n=9), 8 (n=14), 9 (n=9), and 12 (n=2) days, or that were incubated for 7 days with conditioned media from cultures of fetal brain astrocytes (n=7). At all time points, the resting membrane potentials (RMP) of cells co-cultured with astrocytes were variable, ranging between -8.4 and –55.4 mV. However, RMPs tended to become more negative as a function of time in culture with astrocytes. The median RMPs were –27.4, -33.7, -41.9, and –44.4 mV after 5, 7, 8, and 9 days, respectively, in co-culture. Input resistance also varied considerably across cells (range = 0.133 to 9.8 GOhm), however, no trend was apparent in the value of input resistance as a function of time the cells spent in culture with astrocytes (median input resistance = 2.4, 1.6, 2.4 and 1.2 GOhm after 5, 7, 8, and 9 days in culture with astrocytes, respectively).

Current-clamp recordings demonstrated that spiking was observed in cells that were co-cultured with astrocytes at all time points examined. Figure 3A illustrates an example of spiking behavior evoked from a cell that had been co-culture with astrocytes for 8 days. Interestingly, the proportion of cells studied which were capable of generating action potentials increased dramatically after day 5 in culture with astrocytes. Twenty-two percent of cells that were co-cultured with astrocytes for only spiked for 5 days. In contrast, after day 5, spiking cells represented between 71 % and 100 % of cells studied at each time period. Voltage-clamp experiments showed that spiking MAPC expressed a rapidly inactivating inward current. This transient inward current generally could not be elicited from cells, which failed to produce a spike in current-clamp experiments (a small transient inward current was observed in only 1 of the non-spiking MAPC).

Figure 3B shows the inward sodium current, which was elicited from the same cells as in 3A. Approximately 67 percent of spiking cells could be made to spike repetitively in response depolarizing current injection steps; the other 33 percent of cells generated only a single action potential with varying amounts of depolarizing current stimulation. Where examined, the spiking behavior and transient inward currents were blocked by TTX (see panels labeled TTX in figure 3A and B). All cells examined that were co-cultured with astrocytes had outward currents, however, the identities of those currents remain to be determined. Voltage and current traces from our patch-clamp recordings also suggested the occurrence of synaptic events (Figure 3C, see arrows).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the following claims below.

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